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Declarations under Rule 4.17:

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WO 03/002131 A1

(54) Title: STRAIN OF MICRO-ORGANISM LACTOBACILLUS FERMENTUM ME-3 AS NOVEL ANTI-MICROBIAL AND ANTIOXIDATIVE PROBIOTIC

(57) Abstract: The strain of micro-organism *Lactobacillus fermentum ME-3* is a novel anti-microbial and anti-oxidative probiotic. It has a high anti-microbial effect on *Escherichia coli, Shigella sonnei, Staphylococcus aureus, Salmonella typhimurium*, and moderate activity against *Helicobacter pylori strains*. The strain of micro-organism possesses Mn-superoxide dismutase and both its lysates and intact cells have high anti-oxidative activity, increasing the glutathione red-ox ratio in blood sera and able to capture toxic hydroxyl radicals. The strain of micro-organism could be used as a probiotic for the production of functional food (yoghurt, cheese) and non-comestibles (tablets, capsules) for the prophylaxis of intestinal and uroinfections, both for the prevention and treatment of chronic diseases, caused by prolonged oxidative stress.

AMENDED CLAIMS

[received by the International Bureau on 06 December 2002 (06.12.02);
original claim 1 amended (1 page)]

The strain of micro-organism *Lactobacillus fermentum ME*-3 DSM 14241 as a novel anti-microbial and anti-oxidative probiotic for use in pharmacy and food industry and in medicine as a preparation resistant to antibiotics for the prophylaxis and treatment of gastrointestinal and uroinfections, also against chronic diseases induced by prolonged high-grade oxidative stress.

STATEMENT

The aim of present invention is to offer a strain of microorganism as a novel anti-microbial and anti-oxidative probiotic for use in pharmaceutical and food industry, also in medicine as an antibiotic resistant preparation for prophylaxis and treatment of gastrointestinal and uroinfections, also against oxidative stress (P.3, line 33-35, P.4, line 1-3)

Concerning the antimicrobial activity (bacteriostatic influence) of the object of invention, the strain *Lactobacillus fermentum* ME-3 expresses anti-microbial effect beside others on *Shigella sonnei, Salmonella typhimurium ja Helicobacter pylori* strains (Page 6, line 21-25; Page 7, Table 2). In addition, the property of *Lactobacillus fermentum* ME-3 to kill the food borne pathogens in milk (bacteriocidic effect) is firstly described (Page 7, Table 2 and line 6-11).

The innate resistance of *Lactobacillus fermentum* ME-3 against antimicrobial preparations (TMP-SMX, ofloxacin, aztreonam, cefoxitin and metronidazole) allows to use it as a preparation accompanying antibiotic treatment in case of gastrointestinal and uroinfections (Page 7, line 17-19; Page 8, line 1-3). This property has not been described elsewhere before.

The unique carbohydrate profile of the cell wall of *Lactobacillus fermentum* ME-3 enables to prevent the adhesion of uropathogenic *Escherichia coli* to the epithelial cells of

the upper urinary tract, a property that makes our strain applicable in the prophylaxis of urinary tract infections (Page 8, line 5-26)and has never been described before.

Concerning the antioxidative activity of the strain *Lactobacillus fermentum* ME-3 as the object of the present invention the different specific, principal and novel parameters were firstly described like expression of MnSOD, high-grade total antioxidative status (TAS, verified by internationally accepted method), principal parametres of glutathione (a signal molecule and central cellular antioxidant) system and the value of glutathione redox ratio (Page, 9 Table 3; Page 10, line 1-11, 15-19, 23-26).

Any antioxidativity (including antiatherogenicity) parameters found in human trials (*in vivo* trials) were not made public elsewhere. Therefore, only in this invention, an influence of consumption of ME-3 on human blood sera specific indices was described (Page 13-14, Table 6) and disclosed the appropriate numerical values. Actually, considering mainly these parameters (significant increase of TAS and oxygen resistance of LDL, lowering the level of oxidized LDL and its diene conjugates altogether indicate improvement of systemic antioxidativity and also significant lowering of cellular oxidative stress) it can be claimed that strain *Lactobacillus fermentum* ME-3 is a novel antioxidative (anti-atherogenic) probiotic (Page 11, line 25-30).

The persistence of the novel strain in gastrointestinal tract after consumption and the beneficial influence on the composition of the intestinal lactobacilli are desribed for the first time (Page 12, Table 5, line 14-22).

Thus the strain of microorganism *Lactobacillus fermentum* ME-3(DSM 14241) figures a novel antimirobial and antioxidative (anti-atherogenic) probiotic for use in pharmacy and food industry, and in medicine as a preparation resistant to some antimicrobials useful for the prophylaxis and as a preparation accompanying antibiotic therapy of gastrointestinal and urinary tract infections, also against chronic diseases (incl. atherosclerosis) induced by prolonged high-grade oxidative stress.

shape located in parallel chains, nonspore, of medium thickness and different length (2x 3-5 μm).

Physiological-biochemical characteristics: MRS broth was suitable for cultivating the microbial strain during 24-48 hours in a 10% CO_2 environment, after which homogeneous turbid growth occurred in the broth. The colonies of micro-organism on MRS agar are white, rounded, with a regular edging.

The optimal growth temperature is 37°C, it multiplies also at 45°C, but it does not grow at 15°C. The optimal growth environment is at pH 6.5.

The negative catalase test, gas production by fermentation of glucose, production of NH_3 from arginine, and lysozyme production are the main properties. During reproduction in milk it produces 1,07% of acid.

The strain with above-mentioned characteristics was identified on the basis of biochemical activity with API 50 CHL System (BioMerieux, France) kit as *Lactobacillus fermentum* (ID% 99.6, T 0.87, only 1 test contra). The following sugars and alcohols were fermented - ribose, galactose, D-glucose, D-fructose, D-mannose, esculine, maltose, lactose, melibiose, saccharose, D-raffinose, D-tagatose and gluconate.

The profile of the metabolites of *Lactobacillus fermentum* ME-3 was characteristic of heterofermentative metabolism, determinated by the gas chromatographic method (Hewlett-Packard model 6890). The profile of fermentation depended on environment of incubation: besides lactic and acetic acids a big amount of succinic acid was produced in a CO_2 environment, but in an anaerobic environment much of ethanol was produced in addition to the above-mentioned substances (Table 1). Both succinic acid and ethanol can strengthen the

stable properties of the microbial strain in milk fermented by this strain.

Table 1. The concentration of acetic acid, lactic acid, succinic acid and ethanol (mg/ml) in MRS media in cultivation of *Lactobacillus fermentum* ME-3 in microaerophilic and anaerobic environment during 24 and 48 h.

Lactobacillus fermentum ME-3	Lactic acid		Acetic acid		Succinic acid		Ethanol	
	24 h	48 h	24 h	48 h	24 h	48 h	24 h	48 h
CO_2 environment	10.6	11.1	0.8	0.9	18.4	19.5	9.8	7.5
Anaerobic environment	8.2	8.8	1.0	1.0	5.7	9.7	7	33.3

Molecular identification.

Molecular identification by ITS-PCR (internal transcribed spacer - polymerase chain reaction) using *Lactobacillus fermentum* ATCC 14931 as the reference strain verified the previous identification with API 50 CHL.

The micro-organism with the above-mentioned properties was deposited in Deutsche Sammlung für Mikroorganismen und Zellkulturen GmbH-s, the registration number of the deposite is DSM 14241 (19.04.2001).

Anti-microbial activity

Lactobacillus fermentum ME-3 expresses a high anti-microbial effect on *Escherichia coli, Shigella sonnei, Staphylococcus aureus, Salmonella typhimurium* 1 and 2, and *Helicobacter pylori* strains *in vitro* (Table 2).

Table 2. Anti-microbial activity of strain *Lactobacillus fermentum* ME-3 on modified MRS-agar, in MRS broth and milk.

Lactobacillus fermentum ME-3	*Escherichia coli*	*Shigella sonnei*	*Staphylococcus aureus*	*Salmonella typhimurium* 1 and 2		*Helicobacter pylori*
MRS-agar	Inhibition zone (mm)					
CO_2 /anaerobic environment	24/22	26/21	20/19	25.8 / 24.7	23.8 / 19.7	13.2/13.1
MRS broth	Decrease of total count (log_{10}) compared with initial count					
	log 6.0	Log 6.7	log 0.8	log 6.3	log 3.8	not determined
Milk	Suppression after different interval of time (24 - 48 h)					
	24 t	32 t	24 t	32 t	48 t	not determined

Using milk fermentation it was possible to show that pathogens inoculated into milk were killed in 24-48 h if milk was fermented with *Lactobacillus fermentum* ME-3. Such property of the strain could help to prevent the multiplication of pathogens in products (yoghurt, cheese) fermented by this strain, and prevent food infections.
Organic acids and ethanol produced by *Lactobacillus fermentum* ME-3 could ensure the high anti-microbial effect of this microbe.

Resistance to antibiotics

According to a disk-diffusion test (BBL Sensi disks) and an E-test (AB Biodisk, Solna) *Lactobacillus fermentum* ME-3 was resistant to metronidazole, ofloxacin, aztreonam, cefoxitin

and TMP-SMX. This allows to use the strain *L. fermentum* ME-3 as a preparation accompanying antibiotic treatment in case of intestinal and uroinfections.

Surface structures of microbial cell

The carbohydrate profile of the surface structure of microbial cells of *Lactobacillus fermentum* ME-3 was determined by lectin typing. The strain of lactobacilli agglutinated with *Griffonia simplifolia* I lectin, which is specific to Gal and GalNAc ligands in the cell wall.

The strain *Lactobacillus fermentum* ME-3 did not react with the following other lectins: *Concanavalin ensiformis* (Con A), *Griffonia simplicifolia* II, *Arachis hypogaea* (PNA), *Vicia sativa* (VSA) and *Tritium vulgaris* (WGA).

Hence the special composition of the glycocalyx of the cell wall of *Lactobacillus* ME-3 became clear with lectin typing, it contained residues of galactose and N- acetyl-galactose-amine. These compounds act as adhesins for engaging the receptors of mucosa on the epithelial cells of the upper urinary tract.

This is a possibility for blocking the mannose-resistant pili of *Escherichia coli* that makes our strain applicable in the prophylaxis of urinary tract infections.

Anti-oxidative properties

Lactobacilli were incubated in a MRS broth (Oxoid Ltd.) for 24 h and centrifuged at 4° C (1500 p/min) 10 min for getting a precipitate, washed with isotonic salt (4°C) and suspended to the density of 1.15% KCl (Sigma, USA). The density of the suspension was at OD_{600} 1.1 10^9 bacterial cells in ml^{-1}).

To get lysates, the cells were disrupted by sonification (B-12 Branson Sonic Power Company, Danbury, Connecticut) in 35

vibrations s^{-1} 10 min in an ice bath and then for 10 min at -18°C. The suspension was centrifuged at 4°C 10000 g/r for 10 min and the supernatant was filtered (MILLEY-GS, sterile, 0.22 µm; Millipore S.A., 67 Molsheim, France) to get a cell-free extract. *Lactobacillus fermentum* ME-3 cells and lysate produced H_2O_2 in a remarkable amount(Table 3).

Table 3. Total anti-oxidative capacity of *Lactobacillus fermentum* strains ME-3 and E-338-1-1 (according to LA and TAS tests), hydrogen peroxide content, glutathione red-ox ratio and activity of superoxide dismutase.

Properties	*Lactobacillus fermentum* ME-3	*Lactobacillus fermentum* E-338-1-1
	Intact cells	**Intact cells**
TAA in LA-test (%)	29 ± 0.7 (n=5)	0
TAS (mmol/L)	0.16 ± 0.03 (n=5)	0
H_2O_2 (µg/ml)	31 ± 26 (n=3)	49 ± 20 (n=3)
	Lysate of cells	**Lysate of cells**
LA-test (%)	59 ± 3.8 (n=5)	0
H_2O_2 (µg/ml)	229 ± 37 (n=4)	137 ± 25 (n=3)
TGSH	12.5 ± 4.1	5.5 ± 3.0
GSSG (µg/ml)	2.59 ± 2.01	5.5 ± 2.4
GSH (µg/ml)	9.95 ± 3.30	Marks
GSSG/GSH	0.28 ± 0.17	0[e]
SOD (U/mg protein)	0.859 ± 0.309 (n=3)	Not determined

Explanations: LA-test - linolenic acid test; TAA - total anti-oxidative activity; TAS - total anti-oxidative status; GSSG - oxidized glutathione; GSH - reduced glutathione;

GSSG/GSH - glutathione red-ox ratio; SOD - superoxide dismutase

Lactobacillus fermentum ME-3 has a Mn-SOD activity determined by electrophoresis. For determining the SOD type *L. fermentum* ME-3 cell-free extract (30 µg protein) was separated on 10% not-denaturated polyamide-acrylic gel. SOD isoenzyme was determined by influencing this gel with 15mM H_2O_2, after which the SOD activity persisted. Explanation: H_2O_2 inhibits Fe-SOD, but does not inhibit Mn-SOD. This proves that *Lactobacillus fermentum* ME-3 has Mn-SOD activity.

The strain *Lactobacillus fermentum* ME-3 showes a high TAA (total antioxidative activity) value in a lipid environment on the basis of a linolenic acid test, also a high TAS (total antioxidative status) value in a hydrate environment (Randox kit, UK). In Table 3, data of the anti-oxidative strain *Lactobacillus fermentum* E-338-1 is added for comparison (Table 3).

The cells and lysates of the strain *Lactobacillus fermentum* ME-3 catch hydroxyl radicals, this has been proven by the terephthalic acid method (27% ± 5%). 15mM of reduced glutathione was used for comparison as a well-known scavenger of hydroxyl radicals (84±4,6%). *Lactobacillus fermentum* ME-3 survived in a highly oxidative H_2O_2 environment.

Re-cultivation of the lyophilised culture kept in room temperature for a long time proved the viability of the strain and the persistence of properties. This ensures that the lyophilised strain of *Lactobacillus fermentum* ME-3 could be used as a non-comestible product in a scheme of functional food.

BEST MODE FOR CARRYING OUT THE INVENTION

An example of the preparation of a yoghurt with highly anti-oxidative properties based on the strain *Lactobacillus fermentum* ME-3 and the trial of consuming the yoghurt by healthy volunteers.

Lactobacillus fermentum ME-3 pure culture in 0.15% MRS-agar is used for producing the yoghurt, additionally the pure cultures of *Lactobacillus plantarum* and *Lactobacillus buchneri* are seeded into fresh goat milk autoclaved for 20 min at 110°C. Three cultures of these strains of lactobacilli are mixed in equal proportions together with 2% of *Streptococcus thermophilus* and are added in 0.2% of content into autoclaved goat milk.

Lactobacillus fermentum ME-3 with strains of lactobacilli and streptococci will guarantee tasty and highly anti-oxidative yoghurt (Table 4).

Table 4. The anti-oxidative activity of *Lactobacillus fermentum* ME-3 pure culture and probiotic yoghurt

Strain	Total anti-oxidative activity (TAA %)	
	Cells	Yoghurt
Lactobacillus fermentum ME-3	29	70

In tables 5 and 6, the changes of the intestinal micro-flora and indices of oxidative stress of blood sera of healthy volunteers are shown before and after taking the probiotic goat milk yoghurt during 3 weeks. These changes prove the anti-oxidative (incl. anti-atherogenic) effect on human organism.

Even a higher total anti-oxidative activity of goat milk yoghurt compared with the total anti-oxidative activity of intact microbial cells of *Lactobacillus fermentum* ME-3 is shown in table 5.

Additive microbial strains ensure the standard acidity and consistence of yoghurt.

Table 5. The changes of intestinal micro-flora of healthy volunteers (n=16) before and after consuming probiotic goat milk yoghurt during 3 weeks

	Before		After	
	Persons colonised	Lactoflora ratio (%)	Persons colonised	Lactoflora ratio (%)
Consuming goat milk yoghurt (n=16 persons)				
L. fermentum	4*	0,7 – 5,77	16*	0,5 – 49,9#
Taking goat milk (n=4 persons)				
L. fermentum	0	0	1	0 - 32,9

Statistically significant increase:

* The Fisher Exact Test showed the difference of counts in persons colonised with *Lactobacillus fermentum* ME-3 - $p<0,0015$;

The Mann-Whitney Rank Sum Test showed the difference of relative share of *Lactobacillus fermentum* ME-3 in lactoflora.

Therefore, after consuming yoghurt in 3 weeks the microbe was present in the intestinal tract of all volunteers and the *Lactobacillus* sp. count was remarkably increased.

Table 6. The indices of oxidative stress of blood sera of volunteers (n=16) before and after consuming probiotic goat milk yoghurt during 3 weeks

Properties	Standard degree	Blood sera before trial	Blood trial after trial	Increase
TAA (LA-test, %)	36± 4.5	38 ± 3.5	45 ± 3.4	**16%**
TAS, mmol/L	1.2 ± 0.2	0.82 ± 0.14	1.14 ± 0.08	**29%**
Glutathione red-ox ratio (GSSG/GSH)	0.17±0.08	0.15 ± 0.01	0.11 ± 0.035	**-32%**
LDL lag-phase (time of resistance)	>30 min	41 ± 7.9	46 ± 8.6	**11%**
Basic value of diene conjugates (value of extinction)	< 0.3	0.27 ± 0.06	0.23 ± 0.06	**-15%**
Ox LDL (U/L)	>127	98±12	81 ±19	**- 18%**

Explanations: LA-test - linolenic acid test; TAA - total anti-oxidative activity; TAS - total anti-oxidative status; GSSG - oxidized glutathione; GSH - reduced glutathione; GSSG/GSH - glutathione red-ox ratio, ox LDL - oxidized low-density lipoproteins.

Thus all parameters determined in the blood sera of healthy volunteers changed beneficially during the 3-week yoghurt trial.

The application of the invention is not limited to the above-described example of achievement. In the range of the patent claim, some other variants of use are possible, for example the production of probiotic cheese and other milk products.

CLAIM

The strain of micro-organism *Lactobacillus fermentum ME*-3 DSM 14241 as a novel anti-microbial and anti-oxidative probiotic for use in pharmacy and food industry and in medicine as a preparation resistant to antibiotics for the prophylaxis and treatment of intestinal and uroinfections, also against oxidative stress.

Applicant's or agent's file reference	International application No. PCT/EE02/00006

INDICATIONS RELATING TO DEPOSITED MICROORGANISM OR OTHER BIOLOGICAL MATERIAL

(PCT Rule 13*bis*)

A. The indications made below relate to the deposited microorganism or other biological material referred to in the description on page 4, line 5-11.

B. IDENTIFICATION OF DEPOSIT Further deposits are identified on an additional sheet ☐

Name of depositary institution

DSMZ - Deutsche Sammlung von Mikroorganismen and Zellkulturen GmbH

Address of depositary institution *(including postal code and country)*

Mascheroder Weg 1b
D-38124 Braunschweig
Germany

Date of deposit	Accession Number
19.04.2001	DSM 14241

C. ADDITIONAL INDICATIONS *(leave blank if not applicable)* This information is continued on an additional sheet ☐

D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE *(if the indications are not for all designated States)*

E. SEPARATE FURNISHING OF INDICATIONS *(leave blank if not applicable)*

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INTERNATIONAL SEARCH REPORT

ational Application No
PCT/EE 02/00006

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 A61K35/74 C12N1/20 A61P31/04 A61P1/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 A61K C12N A61P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
BIOSIS, EPO-Internal, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	MIKELSAAR MARIKA ET AL: "Antagonistic and antioxidative activity of lactobacilli and survival in oxidative milieu." AMERICAN JOURNAL OF CLINICAL NUTRITION, vol. 73, no. 2S, February 2001 (2001-02), page 495S XP001105810 International Symposium on Probiotics and Prebiotics;Kiel, Germany; June 11-12, 1998 ISSN: 0002-9165 abstract	1
A	SEPP E ET AL: "Intestinal microflora of Estonian and Swedish infants." ACTA PAEDIATRICA, vol. 86, no. 9, 1997, pages 956-961, XP001105447 ISSN: 0803-5253 the whole document	1

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[X] Further documents are listed in the continuation of box C. [ ] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier document but published on or after the international filing date
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"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
23 September 2002	11/10/2002

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Didelon, F

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	KULLISAAR TIIU ET AL: "Two antioxidative lactobacilli strains as promising probiotics." INTERNATIONAL JOURNAL OF FOOD MICROBIOLOGY, vol. 72, no. 3, 2002, pages 215-224, XP002214344 ISSN: 0168-1605 abstract table 1 page 223, column 1, last paragraph	1
P,A	MIKELSAAR MARIKA ET AL: "Intestinal lactobacilli of Estonian and Swedish children." MICROBIAL ECOLOGY IN HEALTH AND DISEASE, vol. 14, no. 2, June 2002 (2002-06), pages 75-80, XP001105478 June, 2002 ISSN: 0891-060X abstract table 2	1
P,A	ANNUK HEIDI ET AL: "Characterisation and differentiation of lactobacilli by lectin typing." JOURNAL OF MEDICAL MICROBIOLOGY, vol. 50, no. 12, December 2001 (2001-12), pages 1069-1074, XP002214345 ISSN: 0022-2615 abstract tables 2,3	1

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.
PCT/AU03/01176

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document Cited in Search Report		Patent Family Member					
WO	0234273	AU	13642/02	AU	14172/02	CA	2426672
		EP	1335736	WO	0235233		

END OF ANNEX

INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU03/01176

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 02/34273 A1 (ATHEROMASTAT PTY LTD) 2 May 2002	1-32